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Regenerative medicine for insulin deficiency: creation of pancreatic islets and bioartificial pancreas.

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Abstract

Recent advances in pancreas organogenesis greatly improved the understanding of cell lineage from inner cell mass to fully differentiated β-cells. Upon such knowledge, insulin-producing cells similar to β-cell to certain extent are generated from various cell sources including embryonic stem cells (ESCs) and induced plurimotent stem (iPS) cells, although fully differentiated cells comparable to β-cell are not yet available. Bioartificial pancreas is a therapeutic approach to enable allo- and xeno-transplantation of islets without immune. Among several types of bioartificial pancreas, micro-encapsulated porcine islets are already used in clinical trials and, perhaps, replace islet transplantation in the near future. Some types of bioartificial pancreas such as macro-encapsulation are also useful to keep transplanted cells to be enclosed for possible need of retrieval. Therefore, early clinical applications of artificially generated β-like cells, especially those from ESCs or iPS cells, will be considered in combination of retrievable BAP.

Introduction

Diabetes mellitus (DM) is a heterogenous metabolic disease that is eventually attributed to insulin deficiency, which is especially true in type 1 diabetes mellitus (T1DM) in which insulin producing β-cells residing in islets of Langerhans of the pancreas are primarily destroyed by autoimmune response or other unknown causes. Therefore, patients of T1DM need insulin replacement or endogenous β-cell reconstruction if exogenous insulin cannot maintain proper blood glucose control. The latter is currently achieved only by either pancreas or islet transplantation. However, these transplantation therapies share common problems of donor scarcity and adverse effects of immune suppression.

The number of people suffering from DM in the world is estimated to be 285 million in

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2010 and to become 439 million in 2030 [1]. This estimation and many other predictions are warning about the possibility that increasing number of DM patients will cause serious socio-economical burden unless effective and efficient therapies are developed for cure and prevention of DM.

Regenerative medicine is the process of creating living, functional tissues to repair or replace tissue or organ function lost due to age, disease, damage, or congenital defects [2]. In DM, especially in T1DM, what is lost is β-cell function. Therefore, regenerative medicine for DM is to create endogenous β-cell function. Toward this end, many possible approaches, ranging from *in situ* regeneration of islet of Langerhans to mechanical artificial pancreas [3], are explored. Among these approaches, remarkable advancements in *ex vivo* creation of cells with β-cell-like function (β-like cells) from various cell sources are recently achieved and, with great expectations, may lead to cellular therapy for T1DM. This article will briefly review these advancements. And then, recent state of bio-artificial pancreas (BAP) will be also reviewed, since BAP that enables cell transplantation without immune suppression should be an essential technology for ideal cellular therapy using *ex vivo* created β-like cells.

In vivo differentiation and growth of β-cell

Studies in developmental biology, using knockout animals and lineage tracing, have revealed how β-cell develops *in vivo*. Several important steps are summarized in Figure 1, which was modified from a figure in our old review article [4]. In these, pancreatic and duodenal homeobox-1 (Pdx1, also known as IPF1, STF1, and IDX1) is a master gene for the pancreas, since Pdx1-ko mice lack the pancreas, and also an important transcription factor for insulin [5]. Neurogenin 3 (Ngn3, also known as Math4B and Atoh5) is an essential transcription factor for pancreatic endocrine cells. Ngn3-positive and hormone-negative cells, although such cells were hardly observed in adult animals, were observed within or adjacent to pancreatic ducts in embryonic stages, and considered to be a common progenitor of all islet cells [6,7]. Recently, Ngn3-positive cells were found and isolated in partial duct ligation model of adult mice [8], suggesting islet neogenesis in adult pancreas as discussed later. In addition to Pdx1, other factors including MafA and Nkx2.2 play important roles in expression of β-cell-specific genes as well as insulin. Therefore, these transcription factors are important to maintain β cell function [9].

Islet tissue shows approx. 20-fold growth after birth in rodents and humans, and it also shows additional compensatory growth in response to increased demand, e.g. obesity and pregnancy [10]. In addition, significant pancreatic regeneration occurs after

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major pancreatectomy (Px) [11, 12] and some types of injury in rodents.

Until recently, there was a long discussion about which cells are responsible for the growth and regeneration of islets and, more fundamentally, whether formation of new islets (neogenesis) really occurs or not. However, recent lineage tracing experiments are expected to put an end to this discussion. Dor et al. generated a transgenic mouse strain in which insulin promoter drives the expression of tamoxifen-dependent Cre recombinase and, further, Cre activates a reporter gene. Thus, in this mouse, reporter gene is only expressed in insulin-expressing cells present at the pulse of tamoxifen, as well as their progeny. They have shown that all β-cells at the observation time point used to be or progeny of β-cells present at the tamoxifen injection, suggesting that adult pancreatic β-cells are formed by self-duplication (replication) in normal life-time and even after minor (70%) Px [13]. On the other hand, as mentioned above, Xu et al. have found Ngn3-positive cells in ductal lining of adult injured pancreas and shown that these cells can become islet cells both in situ and in vitro [8]. In addition, Bonner-Weir and her colleagues performed duct-specific lineage tracing experiments using carbonic anhydrase II (CAII) as a marker to test their hypothesis that mature duct epithelial cells are pancreatic progenitor. Their results show that formation of both new islets and newly differentiated acini from CAII-expressing ductal progenitors occurs during neonatal period [14, 15] and that substantial number of islets are genetically marked in duct ligated pancreatic lobe [14]. Acinar cell-specific lineage tracing of similar technique have been reported [16, 17]. According to these reports, acinar cells contribute to metaplastic duct formation in pancreatitis models but do not become a β-cell after Px, exendin-4 treatment, duct ligation, or chemical pancreatitis. Taken together, new β-cells seem to be formed in adult pancreas either through replication of pre-existing β-cells or islet neogenesis from duct epithelial cells depending on circumstances, but not from acinar calls (Fig 2). Very recently, Solar et al. expressed their negative view for islet neogenesis from the duct in their report dealing with Hnf β–positive cells that appear early in pancreatogenesis and gradually restrained during gestation [18]. Therefore, neogenesis issue may need to be discussed a little longer until its settlement.

Various cell sources for β-like cells

In vitro treatments can induce insulin-producing cells from wide range of cell sources. A number of reports have indicated that insulin-producing cells similar to β-cell to certain extent can be induced in various type of adult cells derived from bone marrow [19], umbilical cord blood [20], liver [21], intestine [22], and so on [4, 23], after introduction of

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pancreatic lineage-related genes such as Pdx1. Protein transduction is another method to change cell fate as well as cell function. Protein transduction of Pdx1, BETA2/NeuroD and other factors are shown to enhance differentiation to β-cell [24]. In addition to adult stem cells, Wei et al [25] showed that human amniotic epithelial cells can produce insulin mRNA under nicotinamide stimulation *in vitro* and can produce human insulin in several weeks when implanted in streptozotocin-diabetic mice. These studies indicate that various cell types can be used for generate β-like cells, although these generated cells are not so mature as β-cell in most cases and there remains safety concern if they are gene-engineered.

Pancreas-derived cells are other cell sources since exocrine pancreatic tissue is a waist after islet isolation for transplantation. Ramiya et al. [26] have reported that pancreatic duct epithelial cells obtained from adult non-obese diabetic (NOD) mice can continuously produce islet-like cell clusters after long term culture and that hyperglycemia can be controlled by transplantation of these clusters. Following this, similar *in vitro* generation of β-like cells from cultured duct-like cells has been reported from several other groups (please refer to the other reviews [27, 28]. In addition, this differentiation is shown to be mediated, at least partly, through phosphatidylinositol-3 kinase-related pathway [29].

As mentioned earlier, acinar cells are shown not to contribute to endocrine cell growth *in vivo*, but they are shown to generate β-like cells *in vitro* through formation of cell cluster in suspension culture [30]. Furthermore, recent report showed that exocrine cells can be directly converted to β-cells in vivo by introducing three transcription factors, Pdx1, Ngn3 and MafA [31]. In addition to duct and acinar cells, cells showing less differentiated fibroblast-like appearance can be cultured from pancreatic tissue. Baertschiger et al. showed that these highly proliferative cells express transcription factors implicated to β-cell development as well as several mesenchymal stem cell (MSC)-specific markers, yet it is not clear whether these cells can re-differentiate to β-like cells [32]. Taken together, pancreatic duct-like cells, and even acinar cells, seem potential source for β-like cells, although the details of these phenomena are mostly unknown and, in general, the growth of these cells is slow. In this aspect, MSC-like cells from the pancreas, with high proliferative ability, may be another candidate.

Embryonic stem cell (ESC) is an attractive cell source for any kind of cell therapies, because they proliferate almost indefinitely and has very wide differentiation potency (pluripotency). Since the establishment of ESC [33, 34], many groups have studied β-cell

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differentiation form ESC. In earlier attempts of Lumelsky et al. [35] and many other groups including ours [36], insulin-producing cell clusters were generated through nestin-positive cells by step-wise procedures somewhat similar to ones for neural cell differentiation. However, these cells can hardly be regarded as β-like cells after the report by Rajagopal et al. [37], in which insulin-positive cells were shown to result mostly by insulin up-take from the culture medium. It is known that several types of insulin-producing cells other than β-cell appear in certain neuronal cell, yolk sac and liver during embryonic development [38]. Therefore, insulin staining and mRNA expression in these early studies are now considered to be attributed mostly to differentiation toward neuron-like cells or extra-embryonic endoderm [39], although our study showed insulin granules by electron microscope, mRNA expression of pancreatic genes such as glucagon and amylase as well as insulin and, further, significant decrease in blood glucose after transplantation to diabetic mice [36]. Anyway, lessons from these earlier attempts emphasize the importance of understanding developmental biology about pancreas and β-cell.

Assady et al. reported generation of insulin-secreting cells from ESC after spontaneous differentiation, although the number of such cells and insulin content in them was low [40]. However, their study clearly showed the possibility that β-like cells can be generated from ESC, and subsequent studies employed strategies that closely follow the differentiation steps toward β-cell during embryogenesis, namely ESC definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm, endocrine presursor and finally islet of Langerhans. Following these multiple steps, D'Amour et al. generated cells that release C-peptide in response to multiple secretory stimuli from human ESCs, but they were only minimally responsive to glucose [41]. Therefore, their methods still need some steps that induce final differentiation of β-cells, e.g. second wave expression of Pdx1 and MafA expression. In the sasme line of study, Kroon et al. recently reported that pancreatic endoderm derived from human embryonic stem (hES) cells efficiently generates glucose-responsive endocrine cells after implantation into mice [42]. In this study, pancreatic endoderm-like tissue that contains few hormone-expressing cells was generated in vitro and then transplanted into epididymal fat pad to allow further development in vivo. Then, the graft became fully responsive to glucose 3 months after transplantation. So far, the factors that induce final β-cell differentiation are unknown, but may be simply time longer than allowed for the *in vitro* protocols, or difficult to reproduce *in vitro*, such as vascularization and the interaction with adjacent tissues [43]. Several other groups also reported *in vitro* differentiation of β-like cells from human ESCs, through definitive

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endoderm with distinct methods [44, 45].

Induced pluripotent stem (iPS) cell [46] is an artificial cell type that is generated from somatic cells to mimic ESC. Human iPS cells avoid the ethical difficulties regarding the use of human embryos and tissue rejection following transplantation if they are generated from patients' own cells. The protocols used in humsn ESCs, iPS cells are also shown to differentiate into β-like cells *in vitro* [47, 48]. ESC and iPS cells are potential and indefinite cell source for cell therapies. However, further studies are needed toward the final differentiation of β-like cells as well as to establish acceptable safety until clinical usage.

Bioartificial pancreas

The original concept of BAP is to enable islet transplantation without immune suppression by protecting islets with semipermeable barrier against immune rejection (Fig 3). BAP can be used not only allotransplantation but also xenotransplantation of the islets depending on the immuno-isolation property of the barrier.

As described above, artificial generation of β-like cells are intensely investigated. If any of these studies reach the end to supply sufficient amount of β-like cells of sufficient quality, transplantation of these cells, as well as human islets, will be regarded as a possible treatment for T1DM. However, one major difficulty in this approach is that the transplanted cells are exposed to the patient's inflammatory and autoimmune environment, which originally destroyed their own β-cells. Therefore, even if a good source of β-like cells can be identified for transplantation therapy, these cells need to be protected against these destructive influences. Therefore, BAP is expected to play an important role in future cellular therapy for T1DM.

BAP can be classified into several types, namely diffusion chamber, blood perfusion and encapsulation types (Fig. 4). Each type has its advantages and drawbacks as described below.

In diffusion chamber type, islets are contained in the space surrounded by semipermeable membrane. Typically, islets are contained between two semipermeable membranes placed on both side of ring-like structure [49]. In addition, islets placed in hollow fibers can be regarded as a particular type of this kind. The structure of this type is rather simple and you can choose optimal membrane with desired pore size. On the other hand, islets tend to clump each other and undergo central necrosis. To place islets with some types of hydrogel such as agarose can avoid this clumping and improve BAP function [50]. Our group has developed mesh-reinforced polyvinyl alcohol (PVA) tube and bag of this type [51]. Recently, Yang et al. developed a chamber implantable to bone

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marrow cavity using calcium phosphate cement as immunoisolative device to enclose insulinoma/agarose microspheres [52] and reported therapeutic used of this to spontaneously diabetic cat [53].

Blood perfusion type is similar to dialysis device. Blood is perfused in the hollow fiber and islets are placed around the fibers. The advantage of this type is high diffusive exchange rate, however this type is hardly implantable in a body and needs some anticoagulation treatment. Ikeda et al. developed BAP of this type and showed its effect in pancreatectomized pigs [54].

Encapsulation type can be divided in to two categories, namely micro-encapsulation and macro-encapsulation. In micro-encapsulation, one or a few islets are encapsulated in hydrogel, such as alginate, like micro-beads. This type is favorable for substance exchange because of its large surface area. However, this type is hardly retrievable once they are implanted into the body, for example abdominal cavity. Most famous example of this type is DIABECELL®, a product of a bio-venture company, Living Cell Technologies [55, 56]. It is a porcine insulin-producing cell micro-encapsulated in alginate hydrogel. Micro-beads are implanted into the abdominal cavity of the patients without any immune suppretion. According to their announcement on the internet, Phase I and II clinical trials are now ongoing in Russia and New Zealand. And, so far, beneficial effects, such as reduced HbA1c levels and/or reduced insulin doses including insulin independence are observed without serious adverse effects. Therefore, their clinical trials are showing promising capability of micro-encapulated islets. However, micro-beads implanted in the abdominal cavity are not retrievable. So, this method will not be suitable for the early application of artificially generated β-like cells unless safety of such cells become proven at high levels.

In contrast to micro-encapsulation, macro-encapsulation encloses islets in a lager hydrogel that can be handled macroscopically. Therefore, macro-encapsulated islets are easy to retrieve and seemingly suitable for early use of artificially generated β-like cells, because it can be retrieved if any kind of adverse events such as tumor formation. Our group developed macro-encapsulated islets using agarose-based hydrogel and showed the effect in xenotransplantation into prevascularized subcutaneous site [57, 58]. Another example of this type of BAP is PVA-macro-encapsulated islets. We developed this device through combination of cryo-preservation methods of islets and hydrogel formation from PVA aqueous solution by freezing and thawing [59]. Implantation of this BAP was show to prevent renal dysfunction observed in severely diabetic mice, suggesting reconstruction of basal insulin secretion by BAP is really effective to prevent diabetic complications, at least to certain extent [60].

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Conclusions and prospective

Recent advances in the study of pancreatic organogenesis revealed cell lineage of β-cells and important steps from inner cell mass from which ESCs are established to fully differentiated β-cells. Understanding these informations is important to establish efficient protocol of β-cell differentiation from other cell types, especially from ESCs and iPS cells. β-like cells can be generated from various cell sources with/without gene transfer. However, fully differentiated β-like cells with high insulin production and glucose responsibility comparable to β-cells are not yet available. Further studies on β-cell differentiation are expected to solve this problem. Among various cell sources, pancreas-derived somatic cells might be most promising since even highly proliferative MSC-like cells isolated from pancreas express several pancreas-specific genes suggesting that they stay at certain stage on the way to β-cell differentiation.

BAP that enables islet allo- and xeno-transplantation without immune suppression is a promising therapeutic approach for T1DM. Micro-encapsulated porcine islets are already used in clinical trials and, perhaps replace islet transplantation in the near future. Some types of BAP such as macro-encapsulation are also useful to keep transplanted cells to be enclosed for possible need of retrieval. Therefore, early clinical applications of artificially generated β-like cells, especially those from ESCs or iPS cells, will be considered in combination of retrievable BAP.

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Figure legends

Fig. 1

Schematic over view of pancreas development and important transcription factors involved in this process during embryogenesis (modified from our previous review [4]).

Fig. 2

Schematic relationship and transdifferentiation between pancreatic cell types during regeneration process or in duct-ligated lobes.

Fig. 3

Schematic figure of bioartificial pancreas.

Fig. 4

Schematic figure of several types of bioartificial pancreas.

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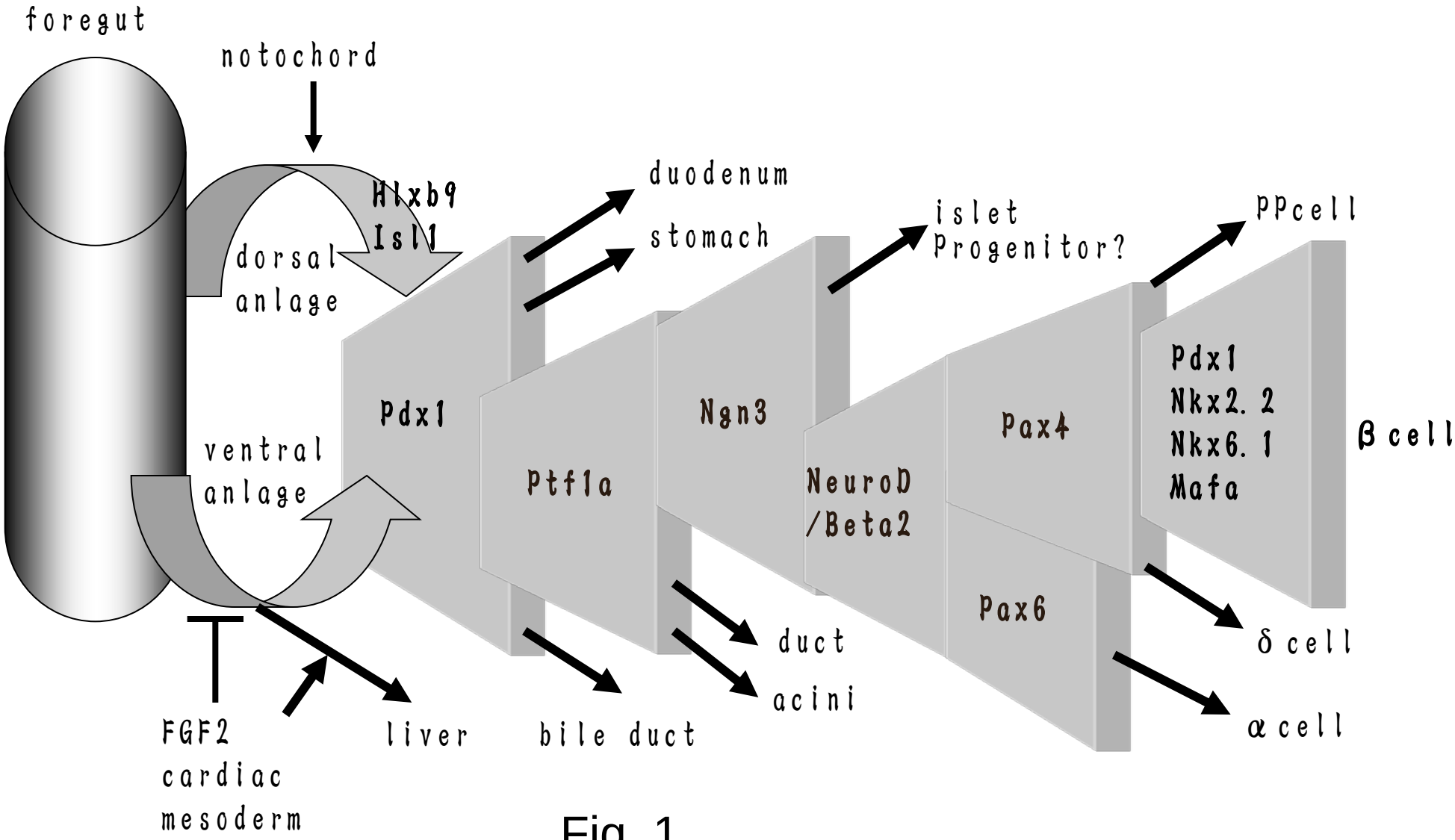

Fig. 1

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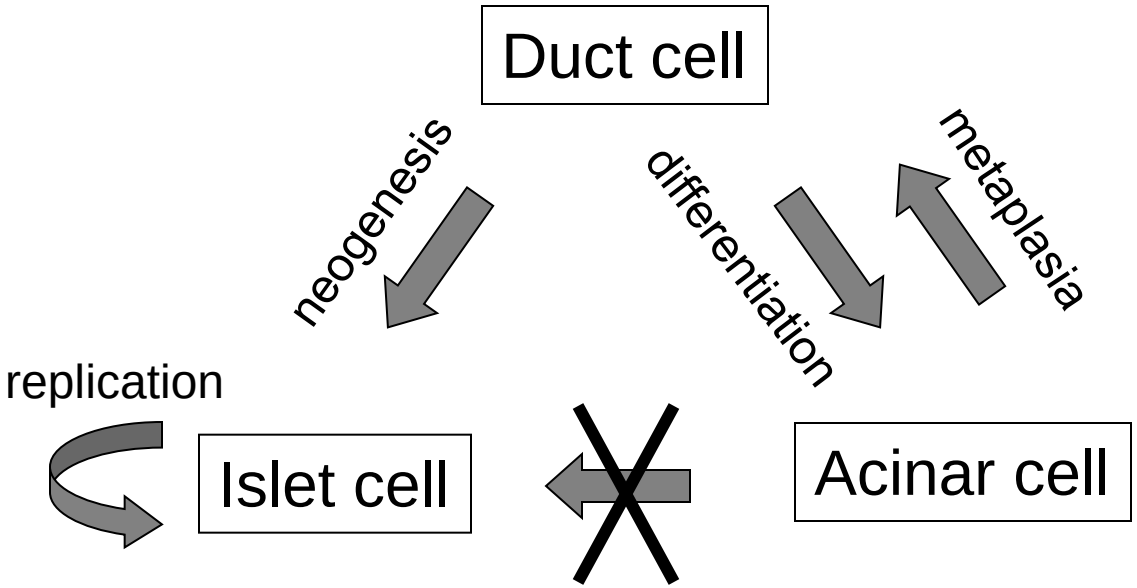

Fig. 2

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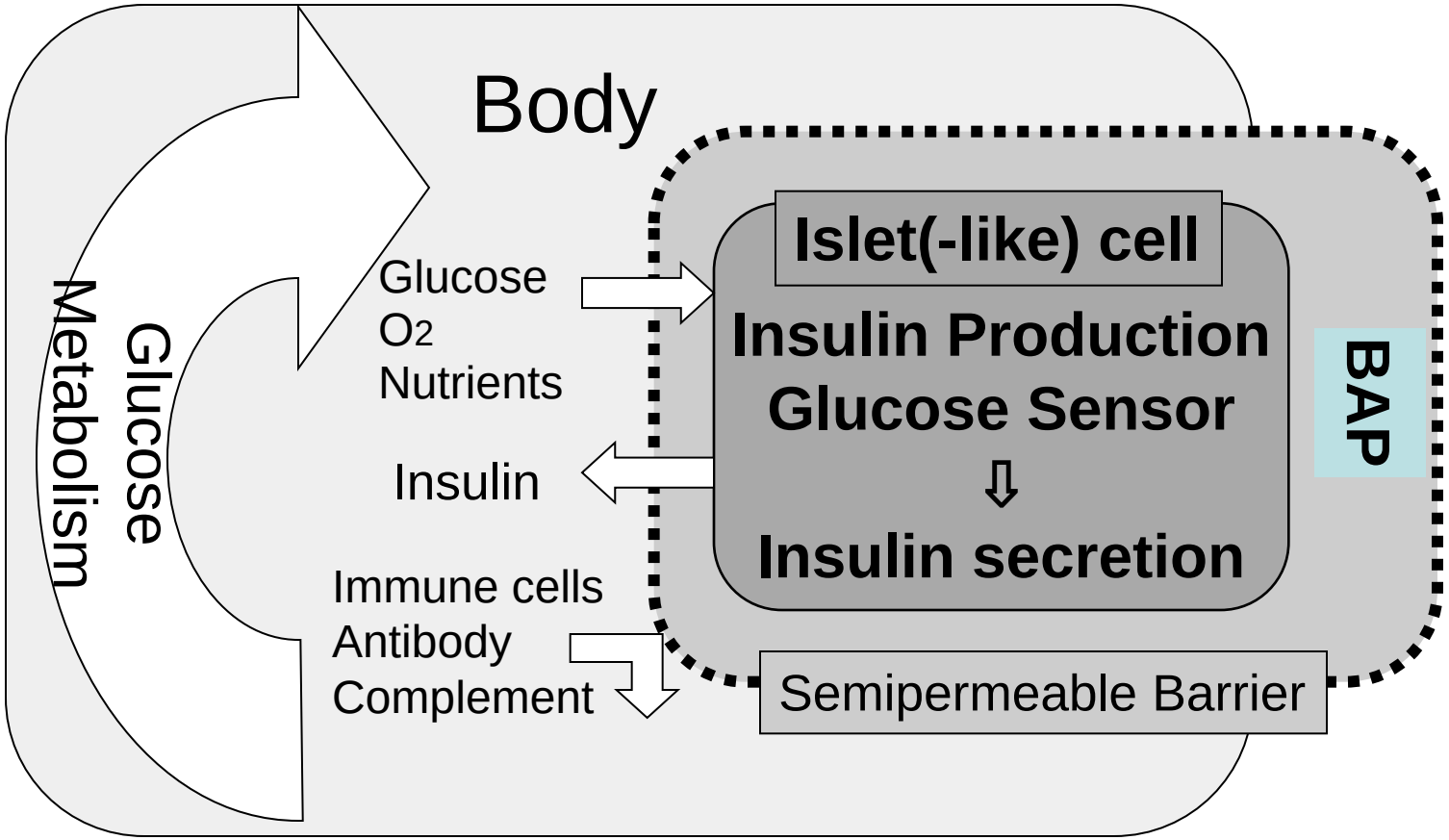

Fig. 3

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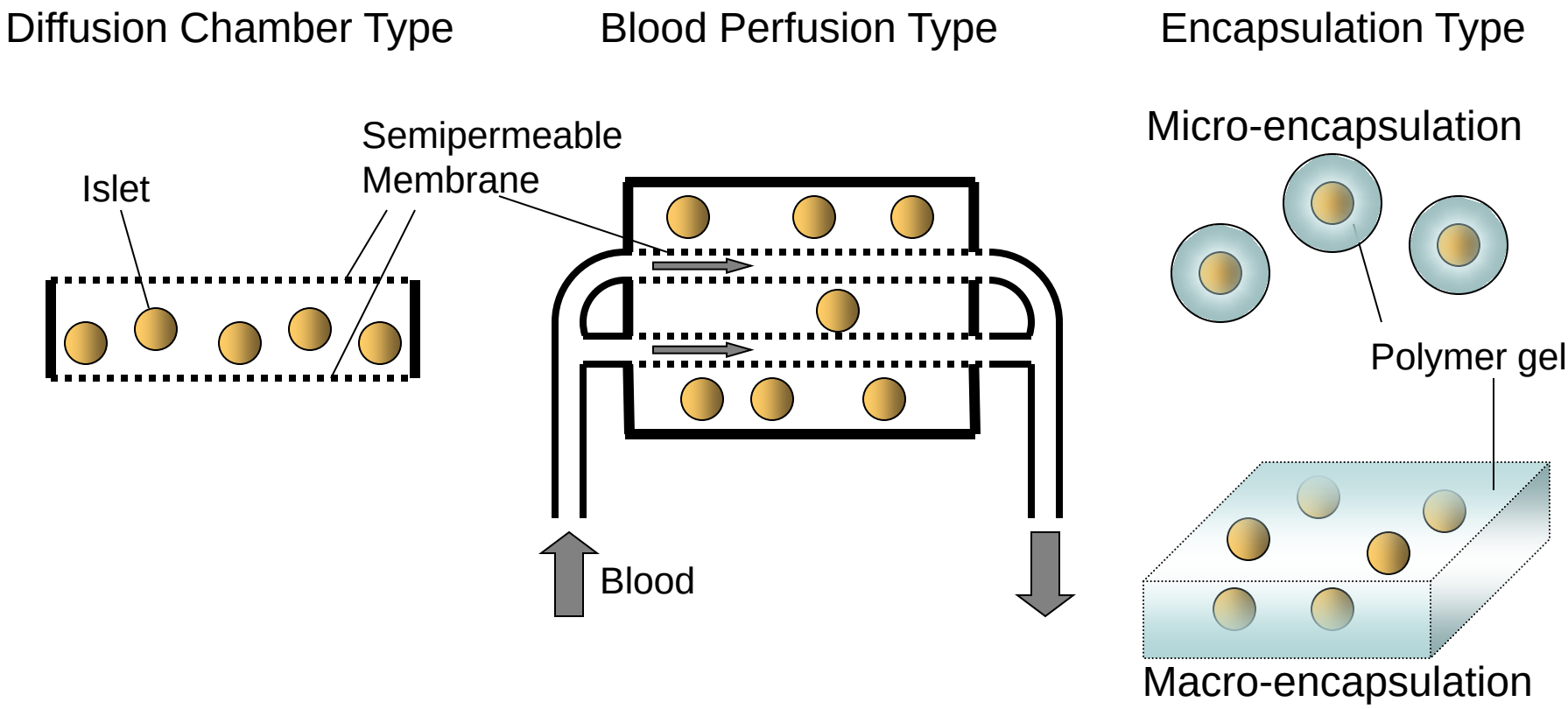

Fig. 4